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(54) Title: DIAGNOSIS OF OCULAR PATHOGENS

(57) Abstract

This invention relates to a method for determining the presence of ocular pathogens in a sample using the polymerase chain reaction and restriction fragment length polymorphism analysis. The method provides a simple way of accurately determining a pathogen to the species level and can detect the pathogen even if only one pathogenic organism is present in the sample.

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DIAGNOSIS OF OCULAR PATHOGENS

Field of the Invention

This invention relates to the diagnosis of ocular pathogens.

Background of the Invention

Ocular infection can result in a profound loss of vision. Infection of the cornea (keratitis) can be broadly divided into that caused by bacteria, fungi and *Acanthamoeba* species, whereas intraocular infection (endophthalmitis) can be either bacterial or fungal. Table 1 illustrates the most common ocular pathogens.

Table 1

Organisms		KERATITIS	ENDOPHTHALMITIS
Bacteria			
Staphylococcus epidermidis	(Gram +)	+	+
Staphylococcus aureus	(Gram +)	+	+
Streptococcus pneumoniae	(Gram +)	+	+
Streptococcus faecalis	(Gram +)		+
Propionobacterium acnes	(Gram +)		+
Bacillus cereus	(Gram +)		+
Pseudomonas aeruginosa	(Gram -)	+	+
Enterobacteriacae	(Gram -)	+	+
Fungi			
Aspergillus fumigatus		+	+
Candida spp		+	+
Fusarium solani		+	
Protozoa			
Acanthamoeba spp		+	

The effect of infection and its consequent inflammation on the eye is considerably worse if appropriate treatment is delayed. In order to maximise the likelihood of a satisfactory visual outcome, broad spectrum intraocular and systemic antibiotics are administered as soon as samples have been collected for culture. This therapy is then adapted once the organism causing the disease is identified by culture. The clinical situation is further complicated by the fact that immune-mediated processes can appear clinically indistinguishable from infection and different organisms such as fungi and bacteria can have very similar clinical appearances. Also, conventional microbial culture techniques have a low yield of positive cultures (around 40%), from cases of presumed

ocular infection, and their results take a minimum of 24 hours and a maximum of 14 days to obtain.

Fungal endophthalmitis is one example of ocular infection and accounts for 4-11% of all cases of culture proven endophthalmitis. It is usually acquired from an endogenous source but may also be secondary to intraocular surgery, corneal ulceration or trauma. The time to diagnosis from the onset of symptoms has been reported as varying from 3 days to 4 months. Fungal endophthalmitis may have a classical appearance e.g *Candida albicans*, but it may also present in a similar manner to chronic bacterial endophthalmitis, or it may coexist with bacterial endophthalmitis. Although *C. albicans* is the causative agent in the majority of cases of culture-proven fungal endophthalmitis, *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. parapsilosis* are increasingly recognised as capable of causing endophthalmitis. As some of these isolates, eg. *C. glabrata*, and *C. krusei*, demonstrate inherent resistance or reduced susceptibility to azole antifungal drugs, it is important to determine the species causing the disease at the first available opportunity. The emergence of non-*albicans* spp as ocular pathogens is of particular concern as very few drugs are available which, when given systemically, achieve adequate vitreous concentrations even in eyes with inflamed blood-ocular barriers. The drugs fluconazole and flucytosine achieve good penetration into the central nervous system and ocular tissues. Unfortunately, their use in clinical therapy is limited due to the resistance to flucytosine which develops very early in treatment and the occurrence of fluconazole resistant strains. Amphotericin B remains the only drug to which resistance has not been encountered in ocular isolates. This drug, however, requires intraocular injection as intravenous administration achieves adequate concentrations only in the retina and choroid but not in the vitreous. As routine clinical management of patients with fungal endophthalmitis involves one intraocular injection of amphotericin B followed by 6 weeks of oral fluconazole therapy, awareness of possible drug resistance to fluconazole is essential. Should resistance occur, the patient will not be receiving the appropriate systemic antifungal therapy to continue treatment for the diseased eye; to treat or protect the fellow eye; or to combat systemic disease. As amphotericin B levels in the vitreous cavity of a vitrectomised eye rapidly fall over a few hours, the protection for the injected eye would also soon come to an end.

Bacterial endophthalmitis is a further example of a severe sight-threatening disease. Early diagnosis and appropriate treatment have been noted to be associated with a better visual outcome, but culture techniques can take between 24 hours and 14 days to confirm the presence and identity of the pathogen. Currently patients are treated with broad spectrum antibiotics as soon as possible after presentation and this treatment is then later customised once results of culture are available. Many cultures, however, prove to be negative, and although this may reflect the purely inflammatory nature of the disease in some cases, it may also be a reflection of the presence of only a small number of bacteria in the sample, some of which may be non-viable following antibiotic treatment and attack from the host's immune defences. This small number may also be a reflection of the difficulties in effective sampling as bacteria can reside in biofilms on intraocular lenses (IOL).

In recent years, polymerase chain reaction (PCR) technology has shown great potential in detecting and identifying low copy numbers of DNA in clinical samples. It also holds great promise in allowing identification of small numbers of organisms in small sample volumes, as is usually the case in ocular samples from patients with endophthalmitis.

However, previous use of PCR to diagnose infection has been too specific, amplifying one or a few particular species, or has been insensitive and not applicable to the small numbers of organisms encountered in samples from patients with keratitis or endophthalmitis.

Summary of the Invention

The present invention relates to a method for the rapid diagnosis of the presence or absence of ocular infection, and the identification of pathogens to the species level.

In particular, the present invention discloses a method for determining the presence of ocular pathogens in a sample, comprising the steps of:

(i) subjecting the sample to conditions under which nucleic acids are extracted;

(ii) amplifying the nucleic acids using a set of bacterial, fungal or *Acanthamoeba* -specific oligonucleotide primers;

(iii) amplifying the product of step (ii) using nested or semi-nested bacterial, fungal or *Acanthamoeba*-specific oligonucleotide primers; and

(iv) detecting any second amplified product.

The method is very sensitive and a diagnosis can be made for fungi even if only one gene copy is present and for bacteria if only one organism is present. Furthermore, the method significantly reduces the time taken to make a diagnosis.

The invention is based on the finding that nested or semi-nested oligonucleotide primers can be used to improve sensitivity in the amplification of pathogen DNA from an ocular sample. Two amplification steps are required: the first requires oligonucleotide primers having broad specificity for ocular pathogens; the second uses nested or semi-nested primers to re-amplify the first step products and to provide genus-specific and in some instances species-specific information. The further use of restriction enzymes may provide species-specific information in the majority of cases.

Detailed Description of the Invention

To ensure that all potential pathogens are detected, the first amplification step uses a pair of oligonucleotide primers designed to amplify highly conserved gene sequences. The primer pairs for bacterial pathogens, fungal pathogens and *Acanthamoeba* species may initially be used together to indicate whether or not an ocular pathogen is present in a sample.

The products from this first amplification step are then re-amplified using nested or semi-nested oligonucleotide primers. It is preferable that these primers also amplify highly conserved gene sequences of bacteria, fungi and *Acanthamoeba* and are genus-specific rather than species-specific. It is therefore possible to identify broadly the organisms most likely causing infection, i.e. either bacterial, fungal or *Acanthamoeba* species, immediately following the second amplification step.

If detection to the species level is required, the products from either the first or second step can be treated with a cocktail of restriction enzymes. Each species will have a particular restriction enzyme map. By using various combinations of restriction enzymes, it may be possible to identify the different species present on the basis of their individual restriction enzyme maps.

The following procedures illustrate the steps that may be taken to isolate the pathogen DNA from a patient with suspected endophthalmitis or keratitis. They are

applicable to each of bacterial, fungal and *Acanthamoeba* pathogens. Alternative procedures will be apparent to those skilled in the art.

Procedure for endophthalmitis cases

Intraocular sampling is routinely undertaken on all patients with suspected endophthalmitis. The extraocular environment was sterilised with 5% Povidone Iodine solution prior to surgery. Approximately 100-200 µl of aqueous fluid was withdrawn using a 27G (0.33mm) needle via a limbal paracentesis. Vitreous samples (200-400 µl) were taken as a biopsy through the pars plana. Ocular samples were examined by Gram and Giemsa staining, and cultured on blood agar, Sabauraud's agar, in Brain Heart Infusion (BHI) and Robertson's Cooked Meat Broth (CMB). Cultures were incubated under both aerobic and anaerobic conditions. All cultures were maintained for 14 days. Isolates were maintained at -70°.

Procedure for keratitis cases

Corneal scrapes were performed at the slit lamp under topical anaesthesia. Sterile 27G needles were used to scrape off sections of corneal epithelium and anterior stroma in the region of the leading edge of the corneal ulcer. Samples were placed on a slide for Gram and Giemsa staining and also plated immediately on Blood and Sabauraud's non-nutrient agar. Plates were incubated under aerobic conditions at 25°C, 30°C or 37°C as appropriate..

Normal Vitreous

Vitreous was collected by sterile technique at the time of vitrectomy during planned surgical procedures in patients with no evidence of intraocular infection or inflammation or medical history of uveitis and/or diabetes mellitus. Samples of normal vitreous were aliquoted in a sterile manner and stored at -20°C.

DNA extraction

1) Fungi

Method 1: Full extraction procedure

DNA was extracted by a method which was modified from that described by Buchman *et al*, Surgery (1990) 108:338-347. DNA extraction tubes were prepared as follows: each tube contained 5 µl of neat vitreous, 100 µl of 0.05 mol/l Tris pH 7.5, 0.01 mol/l EDTA, 0.028 mol/l β-mercaptoethanol containing 0.3mg/ml zymolase and 5 µl of

the required dilution of *C. albicans* in water. Samples were incubated for 30 minutes at 37°C, followed by addition of 0.1% SDS and 15 µg/ml proteinase K, and incubation continued for a further 5 minutes. The mixture was subsequently heated to 95°C for 5 minutes and cooled on ice for 15 minutes. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0), chloroform and precipitated with 2.5 volumes of ethanol in the presence of 300mM sodium acetate. The DNA was pelleted at 14,000g for 20 minutes, washed with ice cold 80% ethanol, air dried at 65°C for 10 minutes and resuspended in 25 µl of sterile TE buffer (10mM Tris, 1mM EDTA, pH8.0).

Method 2: Boil and PCR

The DNA extraction tubes were prepared as follows: each tube contained 5 µl of neat vitreous and 5 µl of the required dilution of *C. albicans* in water. Samples were incubated at 95°C for 20 minutes and subsequently the entire volume was used in PCR reactions.

2) Bacteria

Method 1: Full extraction procedure

Previous work in this laboratory has successfully used glass beads and a bead beater apparatus (Stratech Scientific) to effectively release DNA from bacterial cells in suspension (Hykin *et al*, J. Med. Micro. (1994) 40:408-415). A 2mm colony of bacteria was diluted into 300µl sterile PBS solution, with 50µg/ml proteinase-K and approximately 0.5g glass beads. This mixture was beaten on the bead beater on full power for 10 seconds prior to the addition of phenol (pH 8.0). Samples were vortexed, and centrifuged for 2 minutes at 14,000g in a microfuge and the aqueous phase removed and re-extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform. DNA was then precipitated with 1/10 volume 3M sodium acetate (pH 5.3) and 1 volume isopropanol at -20°C. The DNA was pelleted at 14,000g, for 20 minutes, washed with ice cold 80% ethanol, air dried at 65°C for 10 minutes and resuspended in 25 µl of sterile TE buffer (10mM Tris, 1 mM EDTA, pH 8.0).

Method 2: Boil and PCR:

Bacteria were suspended in the PCR mix and an initial denaturation step of 95° for 5 minutes was added to the PCR cycle.

3) *Acanthamoeba* spp.

Method 1: Full extraction procedure

The method described by Ledee *et al*, Investigative Ophthalmology and Visual Science, (1996) 37:544-550 will be followed. *Acanthamoeba* were lysed in 500µl of UNSET buffer (8 M urea,, 0.15M NaCl, 2% SDS, 0.001M EDTA, 0.1 M Tris pH 7.5). The lysate was phenol-chloroform extracted and the aqueous layer removed and made 0.3M with NaCl. Nucleic acids were then precipitated with two volumes of ethanol.

Method 2: Boil and PCR:

A simplified DNA extraction is reported by Lai *et al*, Molecular and Cellular Probes, (1994) 8:81-89. Incubation of *Acanthamoeba* spp in modified PCR buffer for 1 hour and boiling for 10 minutes was sufficient to release DNA from cells.

PCR assay

Design and optimisation of primers.

Examples of primers used to amplify a highly conserved gene sequence in a pathogen, appear in Table 2. For bacterial pathogens, a suitably conserved gene is the 16S rDNA gene. For yeast pathogens, primers to the cytochrome $P_{450}L_1A_1$ demethylase gene can be used. The 18S ribosomal gene may be used for fungal pathogens. Further embodiments of the invention are as described in the sub-claims. Other suitably conserved genes that may be used as targets for amplifying yeast, bacterial or *Acanthamoeba* DNA, will be apparent to those skilled in the art.

Replitherm Taq (Cambio) DNA polymerase was used for bacterial PCR, whereas, Amplitaq (Perkin-Elmer) DNA polymerase was used for yeast and *Acanthamoeba* PCR.

Table 2

Fungal primers	sequence	Position on the gene sequence
Pan-fungal:		
SEQ ID NO: 1	5'AACTTAAAGGAATTGACGGAA3'	
SEQ ID NO: 2	5'GCATCACAGACCTGTTATTGCCTC3'	
SEQ ID NO: 3	5'GCATATCAATAAGCGGAGGAAAAG3'	
SEQ ID NO: 4	5'GGTCCGTGTTTCAAGACG3'	
SEQ ID NO: 5	AGGGATGTATTTATTAGATAAAAAATCAA	
SEQ ID NO: 6	ATTAATCATTACGATGGTCCTAG	
***Candida* spp:**	Cytochrome P_{450} L_1A_1 demethylase gene	
Outer:		
SEQ ID NO: 7	5'AAGGGGTTATTTATGATTGTCC3'	530-552
SEQ ID NO: 8	5'CCAATACATCTATGTCTACC3'	1540-1560
nested:		
SEQ ID NO: 9	5'ATTGGTATTCTTATGGGTGG3'	1051-1070
SEQ ID NO: 10	5'TTTCAGGGATTCTTAATGGG3'	1302-1321
SEQ ID NO: 11	5'AGTGTTACACAACAGATCAG3'	199-219
SEQ ID NO: 12	5'CATAAGGTTGTTGACCATATG3'	335-355
SEQ ID NO: 13	5'ATTCAACTTATAAAGATGG3'	995-1013
SEQ ID NO: 14	5'CATAACTCAATATGGCTATT3'	
SEQ ID NO: 15	5'CTTTTGACGACATGATTCGA3'	
SEQ ID NO: 16	5'TTGGAGCGGCAGGATAATGG3'	
SEQ ID NO: 17	TAGTGACAATAAATGAC	
SEQ ID NO: 18	CACTCTAATTTTYTCGA	
***Aspergillus* spp:**		
SEQ ID NO: 19	5'GCATTCGTGCCGGTGTACTTC3'	
nested primers		
***Fusarium* spp:**		
nested primers		
SEQ ID NO: 20	CCAATGCCCTCCGGGGCTAAC	
SEQ ID NO: 21	GCATAGGCCTGCCTGGCG	
Bacterial primers		
pan-bacterial:	16S rDNA gene	
outer:		
SEQ ID NO: 22	5'TTGGAGAGTTTGATCCTGGCTC3'	4-25
SEQ ID NO: 23	5'ACGTCATCCCCACCTTCCTC3'	1174-1194
nested:		
SEQ ID NO: 24	5'GGCGGCAKGCCTAAYACATGCAAGT 3'	42-66
SEQ ID NO: 25	5'GACGACAGCCATGCASCACCTGT 3'	1044-1067
SEQ ID NO: 26	GCGRCTCTCTGGTCTGTA	
SEQ ID NO: 27	GTTCCCGAAGGCAC	
SEQ ID NO: 28	TAATCTTGGGGCTTAACCT	

Acanthamoeba primers		
outer:		
SEQ ID NO: 29	5'GGAGCTCCCACGGGAGGCC3'	
SEQ ID NO: 30	5'TGGACCGCGTGAGGCTGCGGCT3'	
SEQ ID NO: 31	GGGCTGCGCTGTGACTACTG	
SEQ ID NO: 32	GTCCTCAAACCTGATATTGG	
nested primers:		
SEQ ID NO: 33	AACCGTGCGGTGGGAAAGTG	
SEQ ID NO: 34	ATTGGGTCAACAAGGCAGAG	

Optimisation for annealing temperature, and magnesium concentration can be carried out to find the primer sets which produce the highest product yield and greatest sensitivity whilst retaining high specificity.

The following examples illustrate the invention with respect to the identification of fungal pathogens (Example 1) and bacterial pathogens (Example 2).

Example 1

Seven novel primers, based on the Cytochrome P450 L_1A_1 demethylase gene were used to detect *Candida* spp and were optimised and formed two nested pairs. Cytochrome $P_{450}L_1A_1$ demethylase was chosen for PCR amplification because its product catalyses an essential step in the conversion of lanosterol to ergosterol. Ergosterol is unique to fungi and yeast cell walls and is not detected in human cells or common bacterial pathogens. Detection and identification of fungi using this gene may also allow more detailed analysis of the active site region by DNA sequencing of resistant and susceptible strains and ultimately the design of primers to detect the presence of specific mutations.

Optimal PCR conditions

Both first round and second round reactions contained 100 µM each dNTP (Pharmacia), 200 µM of each primer, 2.5mM magnesium chloride, 1 unit DNA polymerase 100mM Tris-HCl (pH 8.3) 500mM KCl; 15mM $MgCl_2$; 0.001%w/v gelatin, in a 50 µl reaction. Cycling conditions were identical for both rounds except for the annealing temperature which was found to be optimal at 51.5°C for the first round (primers SEQ ID NO: 7 & 8), 56°C for nested primers I (SEQ ID NO: 9 & 10) and 58°C for semi-nested primers II (SEQ ID NO: 9 & 8). PCR cycling was performed at 95°C for 10 minutes for one cycle, followed by 30 cycles of 94°C for 1 minute, annealing temperature as above for

1 minute, and extension at 72°C for 1 minute. The final cycle was followed by 10 minutes at 72°C.

Electrophoresis and imaging

PCR products were resolved on a 1-2 % agarose/TBE gel according to the size of fragment expected and visualised using ethidium bromide under UV illumination.

DNA sequencing and Restriction maps

Amplified DNA from PCR reactions were purified on agarose/TBE gels, excised, and recovered into solution. PCR fragments were directly cycle sequenced in both directions (using primers SEQ ID NO: 7 & 9) on an ABI prism automated DNA sequencer (model 377 version 2.1.1). Cycle sequencing was repeated 3 times for each product using the same DNA and PCR protocol but different PCR reactions. Sequences were aligned and scanned both manually and with the aid of GCG and REBASE programs provided by the HGMP computer centre. A search was made for a total of 204 restriction endonuclease sites.

RFLP analysis

Following PCR amplification, products were subjected to phenol: chloroform:isoamyl alcohol (25:24:1, pH 8.0) followed by chloroform extraction, and ethanol precipitation. Precipitated DNA was resuspended in ddH_20 prior to digestion with restriction endonuclease enzymes.

Restriction enzyme combinations were used which yielded fragments that allowed easy identification of species following separation of digested DNA on polyacrylamide/TBE gels.

Restriction enzyme digests were performed at 37°C for 2 hours in buffer containing 6mM Tris -HCl, 6mM Mg Cl_2, 150 mM NaCl at pH7.9 and 10 units of each enzyme. The resulting fragments were subsequently resolved on 4-8% polyacrylamide gels and visualised under UV illumination following staining with ethidium bromide.

Specificity of the fungal specific primers

Specificity of the outer primer pair SEQ ID NO: 7 & 8 and semi-nested primers II (SEQ ID NO: 9 & 8) was demonstrated by the amplification of a single 1.0 kb and 500bp fragment, respectively, from all Candida spp tested except *C. guillermondii*. Specificity of the nested primer pair I (SEQ ID NO: 9 & 10) was demonstrated by the

amplification of a single 270bp fragment from *Candida albicans* only. No amplification product was obtained using genomic DNA isolated from *Aspergillus fumigatus, Fusarium solani*, human leucocytes or 13 species of bacteria. The specificity of all the primer combinations is summarised in Table 3, where CA = *C. albicans*, CT = *C. tropicalis*, CP = *C. parapsilosis*, CG = *C. glabrata*, Cpell = *C. pelliculosa*, CK = *C. krusei* and Cgul = *C. guillermondii*.

Table 3

Primers	size/bp	annealing/ 0°C	CA	CT	CP	CG	C pell	CK	C gul
SEQ ID NO: 7 & 8	1030	51.5	+	+	+	+	+	+	+
SEQ ID NO: 9 & 10	270	56	+						
SEQ ID NO: 9 & 8	509	58	+	+	+	+	+	+	
SEQ ID NO: 7 & 10	791	58	+	+					
SEQ ID NO: 14 & 15	244	52	+						
SEQ ID NO: 11 & 12	156	52	+	+					
SEQ ID NO: 14 & 12	218	52	+	+					
SEQ ID NO: 11 & 15	182	52	+	+					
SEQ ID NO: 14 & 10	1184	54	+						
SEQ ID NO: 11 & 10	1122	52	+						
SEQ ID NO: 14 & 8	1423	51.5	+						

Sensitivity of PCR reactions using dilution series of extracted DNA

The sensitivity of the first round PCR was routinely found to be 30-300 pg from dilutions of DNA from *C. albicans* starting from a concentration of 10ng/µl. Assuming a total DNA content of 37fg per organism, and two copies of the gene per diploid genome this is equivalent to 800-8000 organisms. The second round of PCR markedly improves this sensitivity to 10 fg which is equivalent to less than one gene copy. As 10fg is less than the concentration of DNA in one organism, we proceeded to PCR amplify 10fg of *C. albicans* genomic DNA twenty times expecting a positive result one time out of three. A fresh DNA dilution series was prepared using 1.5 µg of *C. albicans* genomic DNA (a concentration at which spectrophotometric DNA concentration measurements are reliable). Two rounds of PCR amplification using SEQ ID NO: 7 & 8 followed by SEQ ID NO: 9 & 10, yielded a positive result from 13 of the 20 tubes (65%). These observed results approximate that which would be expected if one assumes one copy of the gene in every 18.5 fg of genomic DNA would yield a positive result in 54% of the tubes containing 10fg of DNA.

Sensitivity of the PCR with dilution series of live organisms in vitreous

The results obtained using extraction methods one and two were identical. Using method 2, and after first round PCR (with primers SEQ ID NO: 7 & 8), only the control positive was found to have been amplified to such an extent that the 1.0kb product could be detected by ethidium bromide staining of agarose gels after electrophoresis. Subsequent nested PCR reactions demonstrated clear amplification products of the predicted size from all dilutions. Control negatives were included in each protocol and the first round negatives were included as a test sample in the nested PCR reaction. Control negative samples from both rounds of PCR were consistently negative after two rounds of amplification.

Microscopic analysis of dilution series

Microscopic analysis of the numbers of organisms in the most dilute tubes revealed a concordance of 100% between the number of organisms seen in 5 µl of each dilution under the microscope and the number of organisms which grew in culture. This could only be carried out in high accuracy in the more dilute tubes. Microscopy also identified the presence of large clumps of cells (in the more concentrated tubes) which would indicate

poor mixing, revealing the low reliability of pipetting as a mixing process and the low likelihood of achieving similar numbers of organisms in different aliquots of the same dilution fluid. However, the lowest dilution at which organisms could be seen under the microscope, was also the dilution at which the same small number of organisms grew in culture, indicating a high concordance between microscopy and culture in these most dilute samples.

In the dilutions in which culture and microscopy were both negative for organisms but PCR was positive, a variable amount of debris was seen, some of which could possibly have been cellular in origin. Since DNAse treatment of the original colony was performed to destroy all free DNA prior to the preparation of the dilution series, these results would indicate the break up of cells during handling, and the release of DNA into the mixture which may have been PCR amplified but not detected as viable cells by microscopy and/or by culture.

DNA amplification from spiked vitreous

The detection of first round PCR product was successful by ethidium bromide staining of agarose gels in the presence of vitreous if the concentration of vitreous did not exceed 20% of the total reaction volume.

RFLP analysis

The combination of *Alw26I* + *SpeI* successfully differentiated between the 1.0kb PCR amplified products from all 6 yeasts. The use of *Eco RI*+ *Bst XI*+ *Xba I*+ *Nde I* in combination successfully differentiated between the 500bp PCR amplified products from all yeasts. The 500 bp fragment amplified from *C. parapsilosis* genomic DNA remained undigested.

DNA sequencing

The full sequences have been published: C. albicans (Ref.1), *C. tropicalis* (Ref.2), and *S. cerevisiae* (Ref.3) and partial sequences from C. krusei (Ref.4) , *C. glabrata* (Ref.4), *C. parapsilosis* (Ref.5) , *C. kefyr* (Ref.5), and *C. guillermondii* (Ref.5). Genbank accession numbers are AFO19902 and AFO19903 for *C. parapsilosis* and *C. pelliculosa* respectively.

Example 2

PCR-RFLP was performed on eight Gram positive and six Gram negative organisms known to be causative agents of endophthalmitis. Two pairs of oligonucleotide primers based on the 16s rDNA gene were used to PCR amplify a 1.2 kb and a 1.0 kb fragment of bacterial genomic DNA. Restriction fragment length polymorphisms within the PCR product were identified following sequence analysis of the amplified products and subsequently used to speciate the organisms following restriction analysis.

16s rDNA genes are ubiquitous and are present as multiple copies within bacterial genomes. Optimisation for annealing temperature, and magnesium ion concentration were undertaken in an attempt to find the experimental conditions which produced the highest product yield and greatest sensitivity whilst retaining high specificity.

Optimal PCR conditions

PCR reactions contained 20mM Tris-HCl (pH8.3), 100 mM KCl, 0.01% Tween 20, 0.1% NP-40, 100μM each deoxyribonucleotide ('dNTP', Pharmacia Biotech, St Albans, UK), 0.3μM of each primer, 1.5mM magnesium chloride, 3 units Replitherm Taq DNA polymerase ('Taq', Cambio Ltd. Cambridge, UK), in a 25 μl reaction. Both first round and nested PCR cycles commenced with 5 minutes at 95°C. First round PCR with outer primers SEQ ID NO: 22 & 23, was performed by cycling at 94°C for 10 seconds, 56.2 °C annealing for 10 seconds, and 72°C for 15 seconds for 30 cycles. Cycling with nested primers SEQ ID NO: 24 & 25 was performed at 94°C for 7 seconds, 64 °C annealing for 7 seconds, and 72°C for 10 seconds for 30 cycles.

The Taq DNA polymerase used in this study is one of a number of commercially available Taq polymerases with known low levels of DNA contamination. Although this level of contamination is insufficient to give a detectable amplification product after just one round of PCR, it is easily detected following nested amplification. Therefore, in this study, prior to first round PCR amplification, the Replitherm Taq was treated with *AluI* restriction endonuclease (ratio of 3:1 units of Taq: *AluI*, Promega UK Ltd., Southampton, UK), which recognises a specific nucleotide sequence AGCT. The criteria for choosing this restriction enzyme was firstly its known high cutting frequency within the 16s rDNA genomic template, which was evident following sequence analysis of the amplified fragments from the fourteen bacterial species, and secondly the ability to heat inactivate the enzyme prior to addition of template DNA. Prior to PCR amplification the water, PCR

buffer, magnesium and Taq components were mixed and incubated at 37°C with *AluI* for 30 minutes. The restriction enzyme was subsequently inactivated by incubation at 95°C for two minutes, following which the dNTPs, primers, and template DNA were added and the PCR cycle commenced.

DNA sequencing and Restriction Analysis

Amplified DNA from PCR reactions were treated as described for the fungal samples in Example 1, with the exception that the primers used were SEQ ID NO: 22 and SEQ ID NO: 23. A search was made for a total of 268 restriction enzyme recognition sites.

Restriction enzyme analysis

Following PCR amplification, the concentration of PCR product was estimated using ethidium bromide staining of agarose/ TBE gels. Restriction enzyme combinations were used which yielded fragments which allowed easy identification of species following separation of digested DNA on polyacrylamide/TBE gels. Two restriction enzyme cocktails were developed, one to differentiate organisms into Gram positive and Gram negative species (protocol A) and a second cocktail to directly speciate organisms (protocol B).

RFLP protocol A

The restriction enzyme cocktail (5 units each enzyme) was added directly to approximately 1µg of DNA/PCR product in PCR buffer which had been adjusted to contain a final concentration of 2mM Tris HCl (pH8.3). Differentiation into Gram positive and Gram negative species was achieved using restriction enzymes *Sna BI* and *StuI* in combination. The reactions were incubated at 37°C for two hours and halted either by freezing or addition of gel loading buffer containing 10mM EDTA.

RFLP protocol B

The restriction enzyme cocktail (5 units each enzyme) was added to approximately 1µg of DNA/PCR product in PCR buffer which had been adjusted to contain 100mM NaCl, 1mM DTT, and a final concentration of 7mM $MgCl_2$. A restriction enzyme cocktail containing the following nine restriction endonucleases was used to achieve speciation: *AflIII, BssHII, ClaI, DraI, DraIII, HpaI, NdeI, NsiI,* and *SalI*. Restriction enzyme digests

were performed at 37°C for 18 hours The reaction was halted by freezing and restriction fragments analysed on 10% TBE/polyacrylamide gels.

Specificity

Using primers SEQ ID NO: 22 & 23 a 1.2 kb product was obtained from all strains of all species of bacteria tested. When *P.acnes* genomic DNA was used as template, a 600 bp was also obtained and when *P. aeruginosa* genomic DNA was used as template a faint 200bp fragment was usually visible.

Using primers SEQ ID NO: 24 and SEQ ID NO: 25, a single 1.0 kb amplification product was obtained using genomic DNA from all bacteria tested. No amplification product was obtained when genomic DNA from *Candida albicans, Aspergillus fumigatus, Fusarium solani*, or human leucocytes was used as template.

Nested PCR protocol and controls

In each case, 1µl of first round PCR product was added to the nested amplification reaction as template. Control negative (no DNA) samples included water and vitreous (normal or inflamed as appropriate). Control negatives were included in each protocol and the first round negative/s were included as test samples in the nested PCR reaction. Control negative samples from both rounds of PCR were consistently negative after two rounds of amplification.

Control positive samples included extracted genomic DNA (10ng and 10fg) or live organisms (1-5 organisms by dilution from fresh overnight culture) in both water and vitreous. Control positive samples were consistently positive after one round (10ng extracted DNA only) and two rounds of amplification (10fg extracted genomic DNA or 1-5 live organisms).

Sensitivity of PCR reactions

The sensitivity of the first round PCR was routinely found to be 10pg from dilutions of DNA from *E.coli*, coagulase-negative *Staphylococci*, *S. aureus* and *K. pneumoniae* starting from a concentration of 10ng/µl. The sensitivity of the reaction was improved to 1fg following nested PCR. Assuming a total DNA content of 5fg per organism, this is approximately equivalent to a sensitivity of one organism.

To assess the sensitivity of detection with live organisms, serial ten fold dilutions were made from a small 1mm colony of bacteria in water (test organisms: coagulase

negative *Staphylococci* and *E. coli*). Equal aliquots were immediately cultured and amplified in PCR reactions. The sensitivity of detection after one round of PCR was approximately 600 organisms for both species. This sensitivity was improved to one organism after a second round of PCR. The preparations which were the most dilute (10^{-7} - 10^{-12} dilutions) had aliquots which were both culture and PCR positive, both culture and PCR negative, and occasionally PCR positive but culture negative but never culture positive and PCR negative.

DNA sequencing

Each PCR product was sequenced three times. Comparison of the DNA sequences obtained made with the full and partial sequences of the genes already available in Genebank demonstrated that they were derived from the 16s rDNA gene (sequences 97-100% identical). The sequences obtained were submitted to Genebank and have been awarded the following accession numbers: *Streptococcus pyogenes:* AFO76028, *Streptococcus viridans:*AFO76036, *Streptococcus faecalis*: AFO76027, *Propionibacterium acnes*: AFO76032, *Staphylococcus aureus:* AFO76030, *Streptococcus pneumoniae:* AFO76029, *Bacillus cereus:* AFO76031, *Escherichia coli:* AFO76037, *Serratia marcescens:* AFO76038, *Haemophilus influenzae:* AFO76035, *Klebsiella pneumoniae:* AFO76033, *Proteus mirabilis:* AFO76034, and *Pseudomonas aeruginosa:* AFO76039.

Strain testing

A set of control patterns was obtained from all fourteen bacterial species. Subsequently, PCR-RFLP and sequence analysis was performed on all clinical isolates to ensure the reproducibility of the procedure. All clinical isolates of Streptococci, *S. aureus* and each of the *Enterobacteriacae*, results of PCR, RFLP and sequencing were in agreement. RFLP analysis of coagulase-negative Staphylococcal species yielded three patterns. Two of these were still identified as coagulase-negative Staphylococci by a masked observer but a third pattern was found to be un-identifiable. Sequence analysis of clinical isolates of coagulase-negative *Staphylococci* yielded 98% base identity in each case with several of the following *Staphylococcal* species which belong to this group: *S. epidermidis, S. caprae, S. capitis, S. warneri, S. lugdunensis* and *S. pasteurii*. These sequences were also found to be 97% identical to that of *S. aureus*. The converse was also found to be true

of *S. aureus* sequences. These new RFLP patterns have been added to the "control patterns" to aid future identification of these species.

Cloning of PCR products

Sequencing direct from PCR product did not yield adequate sequencing data for the following bacteria: *P.mirabilis*, *H. influenzae*, *S. viridans* and *P. acnes*. PCR fragments were gene cleaned and cloned into pCRII. Subsequent PCR and sequencing was then successfully performed using primers complementary to Sp6 and T7 sites present in the pCRII vector.

RFLP analysis of amplified PCR products from 14 bacterial spp

RFLP analysis differentiates bacterial spp into Gram positive and Gram negative

The use of just two enzymes (*Sna BI* and *StuI*) successfully differentiated between Gram positive and Gram negative organisms. The *Sna BI* and *StuI* recognition sites proved unique to Gram positive and negative bacterial 16s rDNA sequences, respectively, thereby allowing *Sna BI* to cut PCR products derived from Gram positive bacteria only, and *StuI* to cut PCR product derived from Gram negative bacteria. Although the patterns obtained allow differentiation into Gram positive and negative bacteria, speciation was obtained for three bacterial species; *P. aeruginosa*, *H. influenzae* and *P. acnes*.

RFLP analysis differentiates bacterial spp

The combination of *AflIII*, *BssHII*, *ClaI*, *DraI*, *DraIII*, *HpaI*, *NdeI*, *NsiI*, and *SalI* successfully differentiated between the PCR amplified products from 13 of 14 bacterial species. E. coli and S. marcescens yielded identical RFLP patterns and therefore could not be interdifferentiated.

References:

1 Lai MH, Kirsch DR. Nucleotide sequence of cytochrome P450 L1A1 (lanosterol 14 alpha-demethylase) from *Candida albicans*. *Nucleic Acids Res*. 1989; 17: 804

2. Chen C, Kalb VF, Turi TG, Loper JC. Primary structure of the cytochrome P450 lanosterol 14 alpha-demethylase gene from *Candida tropicalis*. *DNA*. 1988; 7: 617-26

3. Kalb VF, Woods CW, Turi TG, Dey CR, Sutter TR, Loper-JC. Primary structure of the P450 lanosterol demethylase gene from *Saccharomyces cerevisiae*. *DNA*. 1987; 6: 529-37

4. Burgener-Kairuz P, Zuber JP, Jaunin P, Buchman TG, Bille J, Rossier M. Rapid detection and identification of *Candida albicans* and *Candida glabrata* in Clinical Specimens by Species-Specific Nested PCR Amplification of a Cytochrome P450 lanosterol -α- demethylase (L1A1) Gene Fragment. *J Clin Microbiol*. 1994;32:1902-1907

5. Morace G, Sanguinetti M, Posteraro B, Lo-Cascio G, Fadda-G. Identification of various medically important *Candida* species in clinical specimens by PCR-restriction enzyme analysis. *J Clin Microbiol*. 1997; 35: 667-72.

CLAIMS

1. A method for determining the presence of ocular pathogens in a sample, comprising the steps of:

(i) subjecting the sample to conditions under which DNA is extracted;

(ii) amplifying the DNA using a set of bacterial, fungal or *Acanthamoeba*-specific oligonucleotide primers;

(iii) amplifying the product of step (ii) using nested or semi-nested bacterial, fungal or *Acanthamoeba*-specific oligonucleotide primers; and

(iv) detecting any second amplified product.

2. A method according to claim 1, wherein the pathogen is fungal, bacterial or protozoan.

3. A method according to claim 2, wherein the pathogen is *Candida* species.

4. A method according to claim 2, wherein the pathogen is a gram +ve bacterium.

5. A method according to claim 2, wherein the pathogen is a gram -ve bacterium.

6. A method according to claim 2, wherein the pathogen is *Acanthamoeba* species.

7. A method according to claim 2, wherein the pathogen is a cause of keratitis.

8. A method according to claim 2, wherein the pathogen is a cause of endophthalmitis.

9. A method according to claim 2, wherein the oligonucleotide primers of step (ii) are selected from those defined herein as SEQ ID NO: 1 to 6, or functional equivalents thereof.

10. A method according to claim 3, wherein the oligonucleotide primers are complementary to nucleotide sequences on the cytochrome P450 L_1A_1 demethylase gene.

11. A method according to claim 10, wherein the oligonucleotide primers of step (ii) are those defined herein as SEQ ID NO: 7 and SEQ ID NO: 8, or functional equivalents thereof, and the oligonucleotide primer of step (iii) are selected from any of those defined herein as SEQ ID NO: 9 to 18, or functional equivalents thereof.

12. A method according to claim 4 or claim 5, wherein the oligonucleotide primers are complementary to nucleotide sequences on the bacterial 16s rDNA gene.

13. A method according to claim 12, wherein the oligonucleotide primers of step (ii) are those defined herein as SEQ ID NO: 22 and 23, or functional equivalents thereof, and

the oligonucleotide primers of step (iii) are selected from any of those defined herein as SEQ ID NO: 24 to 28, or functional equivalents thereof.

14. A method according to claim 6, wherein the oligonucleotide primers of step (ii) are selected from any of those defined herein as SEQ ID NO: 29 to 32, or functional equivalents thereof, and the oligonucleotide primers of step (iii) are those defined herein as SEQ ID NO: 33 and 34, or functional equivalents thereof.

15. A method according to any preceding claim, wherein step (i) comprises incubating the sample at a temperature above 90°C.

16. A method according to any preceding claim, wherein the amplification reaction has an annealing temperature of at least 51.5°C.

17. A method according to any preceding claim, wherein either or each amplified product is treated with one or more restriction enzymes.

18. A method according to any preceding claim, wherein the sample is from the cornea or intraocular fluids.

19. The use of nested oligonucleotide primers capable of hybridising to the cytochrome P450 L_1A_1 demethylase gene, for detecting *Candida* species in samples from the cornea or intraocular fluids.

20. The use according to claim 19, wherein the primers hybridise to the demethylase gene between nucleotides 157-361 and 382-1560 of the said gene.

21. The use according to claim 19 or claim 20, wherein the primers are as defined in claim 10, or the complement thereof.

22. An oligonucleotide primer capable of high stringency hybridisation to a genus-specific sequence of the Cytochrome P450 L_1A_1 demethylase gene between nucleotide numbers 157-361 and 382-1560.

23. An oligonucleotide primer according to claim 22, wherein the primers are as defined in claim 10, or the complement thereof.

24. An oligonucleotide primer capable of high stringency hybridisation to a genus-specific sequence of the bacterial 16S rDNA gene.

25. An oligonucleotide primer according to claim 24, wherein the primers are as defined in claim 13, or the complement thereof.

26. A kit for the detection of ocular pathogens, comprising separate packages of bacterial, fungal or Acanthamoeba-specific oligonucleotide primers and of nested or semi-nested bacterial, fungal or Acanthamoeba-specific oligonucleotide primers.

27. A kit according to claim 26, additionally comprising restriction enzymes.

28. A kit according to claim 26 or claim 27, wherein the oligonucleotide primers are as defined in any of claims 19 to 25.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: The Institute of Ophthalmology
(B) STREET: University College London, 11-43 Bath Street
(C) CITY: London
(D) STATE: N/A
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): EC1V 9EL

(ii) TITLE OF INVENTION: Diagnosis of Ocular Pathogens

(iii) NUMBER OF SEQUENCES: 34

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AACTTAAAGG AATTGACGGA A 21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCATCACAGA CCTGTTATTG CCTC 24

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Olignucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCATATCAAT AAGCGGAGGA AAAG 24

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGTCCGTGTT TCAAGACG 18

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGGGATGTAT TTATTAGATA AAAAATCAA 29

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATTAATCATT ACGATGGTCC TAG 23

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Olignucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAGGGGTTAT TTATGATTGT CC 22

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCAATACATC TATGTCTACC 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATTGGTATTC TTATGGGTGG 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TTTCAGGGAT TCTTAATGGG 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AGTGTTACAC AACAGATCAG 20

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CATAAGGTTG TTGACCATAT G 21

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATTCAACTTA TAAAGATGG 19

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CATAACTCAA TATGGCTATT 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CTTTTGACGA CATGATTCGA 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTGGAGCGGC AGGATAATGG 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TAGTGACAAT AAATGAC 17

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CACTCTAATT TTYTCGA 17

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GCATTCGTGC CGGTGTACTT C 21

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCAATGCCCT CCGGGGCTAA C 21

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GCATAGGCCT GCCTGGCG 18

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TTGGAGAGTT TGATCCTGGC TC 22

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

ACGTCATCCC CACCTTCCTC 20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GGCGGCAKGC CTAAYACATG CAAGT 25

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GACGACAGCC ATGCASCACC TGT 23

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GCGRCTCTCT GGTCTGTA 18

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GTTCCCGAAG GCAC 14

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

TAATCTTGGG GCTTAACCT 19

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GGAGCTCCCA CGGGAGGCC 19

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

TGGACCGCGT GAGGCTGCGG CT 22

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GGGCTGCGCT GTGACTACTG 20

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GTCCTCAAAC CTGATATTGG 20

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

AACCGTGCGG TGGGAAAGTG 20

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

ATTGGGTCAA CAAGGCAGAG 20

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/GB 98/02705

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MCCARTHY M. ET AL.,: "The use of a bacterial ribosomal DNA probe for the detection of a bacteria with possible application to ocular fluids" INVEST. OPHTHALMOLOGY & VISUAL SCIENCE, vol. 30, - 1989 page 198 XP002087523 see the whole document ---	1,2,4,5, 8,24,26
X	KIM K. S. ET AL.,: "Polymerase chain reaction for acanthamoeba keratitis, primers selection and inhibitory factors" INVEST. OPHTHALMOLOGY & VISUAL SCIENCE, vol. 38, no. 4, - 15 March 1997 page s1073 XP002087524 see the whole document ---	1,2,6,26
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1998	30/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MONSTEIN H -J ET AL: "DETECTION AND IDENTIFICATION OF BACTERIA USING IN-HOUSE BROAD RANGE 16S RDNA PCR AMPLIFICATION AND GENUS-SPECIFIC DNA HYBRIDIZATION PROBES, LOCATED WITHIN VARIABLE REGION OF 16S RRNA GENES" APMIS, vol. 104, no. 6, June 1996, pages 451-458, XP000749407 see summary	24
X	WO 96 18745 A (SMITHKLINE BEECHAM CORP ;HOYER LOIS L (US); LIVI GEORGE P (US); SH) 20 June 1996 see whole doc. esp. p.2 line 20ff and claims	1-3,10, 19,26
X	US 5 552 279 A (WEISBURG WILLIAM G ET AL) 3 September 1996 see whole doc ,e.g. claim 10	24
A	WO 95 13396 A (U GENE RESEARCH BV ;FLUIT ADRIAAN CAMILLE (NL); WIDJOJOATMODJO MYR) 18 May 1995 see whole doc, esp.claims 5; incl. seq id.23,	1,2,12, 13,24,26
A	KOWALSKI R.P. ETAL.,: "a comparison of enzyme immunoassay and polymerase chain reaction with the clinical examination for diagnosing ocular herpetic disease" OPHTHALMOLOGY, vol. 100, no. 4, - 4 April 1993 pages 530-533, XP002087522 see the whole document	1-28
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P,X	OKHRAVI N. ET AL.,: "Polymerase chain reaction and restriction fragment length polymorphism mediated detection and speciation of Candida spp causing intraocular infection" INVEST. OPHTHALMOLOGY & VISUAL SCIENCE, vol. 39, no. 6, - May 1998 pages 859-866, XP002087521 see the whole document	1-3,10, 19,22, 26-29

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 98/02705

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